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Abbreviations
abFP, acid brightening Fluorescent Protein, ADBE, Activity-Dependent Bulk Endocytosis, APEX, Ascorbate Peroxidase, BONCAT, Biorthogonal Noncanonical Amino acid Tagging, CME, Clathrin-Mediated Endocytosis, DAB, Diaminobenzidine, dSTORM, direct Stochastic Optical Reconstruction Microscopy, EM, Electron Microscopy, FKBP, FK504 Binding Protein, FLIM, Fluorescence Lifetime Imaging, FRB, FKBP-rapamycin Binding, FRET, Forster Resonance Energy Transfer, GFP, Green Fluorescent Protein, HRP, Horse Radish Peroxidase, iGluSnFR, intensity-based Glutamate Fluorescent Reporter, iSnFR, intensity-based Fluorescent Reporter, miniSOG, mini Singlet Oxygen Generator, mTOR, mammalian Target of Rapamycin, PALM, Photo-Activated Localisation Microscopy, pH, potential of Hydrogen, sdTIM, subdiffractional Tracking of Internalised Molecules, SILAC, Stable Isotope labelling by Amino acids in Cell culture, SILAM, Stable Istope Labelling in Mammals, smFRET, single molecule FRET, SNARE, soluble N-ethylmaleimide-sensitive factor Attachment Receptor, STED, Stimulated Emission Depletion Microscopy, uPAINT, universal Point Accumulation Imaging in Nanoscale Topography, vGAT, vesicular Gamma Aminobutyric acid Transporter, vGLUT, vesicular Glutamate Transporter

Abstract

Over the course of the last few decades it has become clear that many neurodevelopmental and neurodegenerative disorders have a synaptic defect, which contributes to pathogenicity. A rise in new techniques, and in particular –omics based methods providing large datasets, has led to an increase in potential proteins and pathways implicated in synaptic function and related disorders. Additionally, advancements in imaging techniques have led to the recent discovery of alternative modes of synaptic vesicle recycling. This has resulted in a lack of clarity over the precise role of different pathways in maintaining synaptic function and whether these new pathways are dysfunctional in neurodevelopmental and neurodegenerative disorders. A greater understanding of the molecular detail of presynaptic function in health and disease is key to targeting new proteins and pathways for novel treatments and the variety of new techniques available provides an ideal opportunity to investigate these functions. This review focuses on techniques to interrogate presynaptic function, concentrating mainly on synaptic vesicle recycling. It further examines techniques to determine the underlying molecular mechanism of presynaptic dysfunction and discusses methods to identify molecular targets, along with protein-protein interactions and cellular localisation. In combination, these techniques will provide an expanding and more complete picture of presynaptic function. With the application of recent technological advances, we are able to resolve events with higher spatial and temporal resolution, leading research towards greater understanding of dysfunction at the presynapse and the role it plays in pathogenicity.

1. Introduction

Normal brain function relies on efficient neuronal communication, or neurotransmission. Maintaining neurotransmission is essential to ensure the fidelity of information transfer from one neuron to the next and the ability of the cell to respond to the local environment. Neurons are highly polarised cells with specialised synaptic compartments to send and receive information to/from surrounding cells. When this process breaks down, synaptic frailty can result and underpins the "synaptopathy" theory of neurodegeneration whereby disruption of synapse function, whether at the presynapse or the post-synapse, correlates with pathophysiology (Li *et al.* 2003; Lepeta *et al.* 2016).

The current understanding of the molecular mechanisms of neurotransmission have been reviewed in detail (Chanaday *et al.* 2019; Rizo 2018; Chanaday & Kavalali 2018; Lou 2018; Milosevic 2018; Maritzen & Haucke 2018; Soykan *et al.* 2016; Kononenko & Haucke 2015). However, in brief, a depolarising stimulus enters the presynaptic nerve terminal to facilitate the opening of voltage-sensitive calcium channels (Figure 1). Subsequent calcium influx stimulates the coordinated fusion of neurotransmitter-containing synaptic vesicles (SVs) with the plasma membrane in a process termed exocytosis (Südhof 2013). This is aided by the assembly of SNARE proteins that mediate the docking, priming and fusion of the SV with the plasma membrane (Rizo 2018). Neurotransmitter is then released into the synaptic cleft, binding to the post-synaptic nerve terminal of the innervating cell and enabling information transfer.

Following exocytosis, the SV membrane and protein cargo are retrieved via specialised endocytic pathways. Through this process, SVs are reformed and refilled for use in subsequent rounds of exocytosis. This overall process is known as SV recycling and without this ability, neurons would rely on constant *de novo* generation of SVs, which would not be rapid enough to sustain neurotransmission. To date, several modes of exocytosis and endocytosis have been described and are dependent on the neuronal cell type and stimulus the nerve terminal is subjected to (Soykan *et*

al. 2016). For example, during exocytosis SVs can fully fuse and integrate with the plasma membrane or partially fuse in a process called kiss and run. Classically, SVs were believed to be retrieved via clathrin-mediated endocytosis (CME) whereby single vesicles are endocytosed with the aid of a clathrin-coat. During strong stimuli, membrane is retrieved by activity-dependent bulk endocytosis (ADBE) into endosomes which later bud to reform SVs (Chanaday *et al.* 2019). More recently, evidence has emerged for other endosome-mediated pathways such as ultrafast endocytosis (Watanabe *et al.* 2013b) and other clathrin-independent modes of retrieval (Casamento & Boucrot 2020). This has fuelled a renewed interest in understanding the different SV retrieval pathways in health and disease.

The proteins, lipids and pathways involved in SV recycling and neurotransmission have been investigated over the course of the last 70 years and the picture emerging is of a very complex network of protein-protein and protein-lipid interactions which are tightly regulated through a range of different mechanisms. The current challenges are determining which pathway is active under different circumstances and which molecular players mediate these steps. SV recycling has been incredibly difficult to understand given the rapidity of the process (milliseconds to seconds) and the small space in which this occurs as small central nervous system synapses are often only 1-2 um in diameter.

There are many different model systems available to investigate presynaptic function which are comprehensively reviewed by Gan and Watanabe (Gan & Watanabe 2018). The choice of model system will be dependent on the specific requirements of the question being asked. The frog neuromuscular junction was one of the first model systems used (Heuser & Reese 1973; Ceccarelli *et al.* 1973) due to the ease of dissection and is still a popular choice due to the ability to use both optical methods and electrophysiology to monitor function. Additionally the neuromuscular junction in intact organisms such as *C. elegans* and *Drosophila*, can be used for an *in vivo* context. Large, atypical synapses, such as those of the reticulospinal neurons from lamprey, the retinal bipolar synapse, usually from goldfish, and the calyx of Held from the mammalian brainstem are ideal for ultrastructural and electrophysiogical studies due to their size and ability to be directly patched. These synapses are amenable to pharmacological treatments and in the case of the lamprey reticulosynapse have been extensively used in combination with microinjection of antibodies and proteins. Studies using these model systems have provided important insights into presynaptic function, particularly on the kinetics and dynamics of SV recycling.

Mammalian brain slices and cultured neurons from the hippocampus, cortex and several other brain regions are also widespread model systems used to investigate presynaptic function. The neurons in these systems are central nerve terminals which are often the targets of neurodegenerative processes, making them a popular model choice. However, they are generally much smaller than the previously discussed synapses and thus direct patching of these terminals is not possible. The explosion in the availability of genetically modified mouse/rat lines and the ability to manipulate the expression of molecular targets in culture make these systems highly genetically tractable – a key consideration when choosing a model system. Regardless of the model system selected, investigation of presynaptic function requires the use of both novel and established techniques to continually advance our knowledge of the processes occurring at the molecular and cellular level and, crucially, in the context of disease.

Neurological diseases are some of the more debilitating and poorly understood diseases and can occur early in life during development as is the case for intellectual disability or epilepsy, or appear later in life in the form of neurodegenerative disorders, such as Alzheimer's or Huntington's disease. Many proteins implicated in neurological disorders are linked to synaptic function (Waites & Garner

2011). Indeed, identification of mutations in many of these proteins has also informed our ideas of normal presynaptic function and how this impacts on neuronal function in disease. Understanding of the role of synaptic dysfunction is key to early prevention as there is a large body of evidence suggesting that there are changes at the synaptic level before pathogenic changes in cells or detectable symptoms in patients (Lepeta *et al.* 2016).

With the recent development of novel techniques that can be applied to studying presynaptic function and with the increasing impact of neurodegenerative disorders on society, now is an ideal time to develop our understanding of presynaptic function and dysfunction in disease. This review aims to summarise the established and novel techniques available to investigate presynaptic function, with a focus on SV recycling. However, many of the techniques discussed are applicable to the post-synapse and many other cellular processes.

This review will begin by addressing techniques used to investigate the structure and real-time function of the presynaptic nerve terminal, followed by techniques used to identify molecular targets and interactions and finally how using specific labelled targets, high resolution techniques can be utilised to investigate molecular detail. With the evolving technology it is possible to continue to push the boundaries of our knowledge of how the synapse functions in health and disease.

2. Identification of a presynaptic defect

A good starting point to investigate the molecular cause of altered synaptic function is to examine the whole synapse in a non-biased way from a morphological perspective as well as the functional level. This facilitates identification of specific pathways which are functioning aberrantly, providing clues to potential targets for further investigation. This is particularly useful when using a new model of disease where the genetic mutation may be known but not how this translates into dysfunctional synaptic activity. The following sections concentrate on techniques which look more broadly at the synapse as well as monitoring the dynamics and kinetics of synaptic function and the many pathways operational within it.

2.1 Insight into dysfunction: perturbed gross morphology of the pre-synapse

Electron microscopy (EM) has informed presynaptic function since the classical studies by Heuser and Reese and Ceccarelli suggesting different SV retrieval pathways were operational in a nerve terminal (Heuser & Reese 1973; Ceccarelli *et al.* 1973). Over the course of the last 50 years, electron microscopy of nerve terminals has become a standard approach to visualise perturbed presynaptic function. Gross alterations in structure provide an immediate clue to changes in the SV pool size or distribution – seen as reduced numbers of vesicles or more widely dispersed for example following disruption of synapsin function (Rosahl *et al.* 1995; Pieribone *et al.* 1995). Fixation after rounds of stimulation and or recovery can also be used to determine if SV retrieval is functional – if impaired this would be seen as a reduced number of SVs and/or endosomes.

EM is also an effective means to visualise where the SV cycle is perturbed. For example, several key exocytosis proteins were shown by EM to have a role in vesicle docking by quantifying the number and distance of the dense core vesicles from the plasma membrane – removal of these proteins reduced the number of granules close to the plasma membrane and thus informed the likely cause

of reduced exocytosis (Tian *et al.* 2005; de Wit *et al.* 2009; Mohrmann *et al.* 2013; Gowrisankaran *et al.* 2020).

The detail provided by EM also allows analysis of the size, shape and location of retrieving endocytic profiles, whether or not they have clathrin-coat structures around them and if there are invaginating membrane profiles (Cao *et al.* 2017; Gad *et al.* 2000; Zhang *et al.* 1998; Ritter *et al.* 2013; Koenig & Ikeda 1999; Cremona *et al.* 1999; Winther Å *et al.* 2015). The giant squid axon has proven particularly useful for this due to its relatively large size allowing injection of antibodies and peptides to manipulate protein-protein interactions (Shupliakov *et al.* 1997; Ringstad *et al.* 1999; Andersson *et al.* 2010; Morgan *et al.* 2001; Pechstein *et al.* 2020).

Slices through structures provide high resolution detail of a thin section of tissue, however using 3-dimensional reconstructions of nerve terminals provides more detailed context on the location of intracellular structures and whether they are tethered to other membranes (Watanabe *et al.* 2014b; Jiao *et al.* 2010; Wu *et al.* 2014; Tao *et al.* 2018), which is specifically important for retrieving membrane structures. 3D electron tomography reconstructions are generated by a series of 2D images taken of the same sample from different angles which is then combined to give a 3D rendered image (Harlow *et al.* 2001; He & He 2014). These techniques, although undoubtedly powerful to provide a 3D rendering of a nerve terminal, are very time consuming and therefore not high throughput. They are also highly reliant on large amounts of computing power and expertise and as a result are often used to support for a theory.

EM, whether in 2D or 3D, provides information about a snap-shot in time and so therefore is limited in the temporal information it can generate. Used in conjunction with fluid-phase markers, however, endocytic retrieval can be followed. Horse Radish Peroxidase (HRP), is a useful marker for this. It is a small molecule and therefore readily incorporated into the lumen of internalising structures. Conversion to an electron dense product using diaminobenzidine (DAB) oxidation, reveals the internalised structures. Using this technique multiple retrieval pathways can be labelled simultaneously and quantified – for example the extent of ADBE and CME can be quantified by counting the labelled endosomes and vesicles respectively (Clayton *et al.* 2008; Cheung *et al.* 2010). This is a particularly powerful method to examine the relative contributions of different pathways to presynaptic dysfunction in models of disease with unknown protein targets.

EM has been limited by potential artefacts introduced either by fixatives or by the damage caused through the formation of ice-crystals beneath the surface of a sample (Watanabe 2016; Korogod *et al.* 2015; Studer *et al.* 2014). However, Flash-Freeze EM is a new technology combining optogenetic stimulation of neuronal activity with rapid freezing of samples at precise time intervals allowing visualisation of the events occurring at the synapse with millisecond resolution. Freezing under pressure means that thicker sample preparations can be preserved without ice crystal damage therefore expanding the potential of this technique beyond cultured cells and into whole animals, such as *C. elegans*, facilitating investigation of synaptic function *in vivo* (Watanabe et al. 2013a). Flash-freeze EM was instrumental in the identification of ultrafast endocytosis, a discovery which has changed the field's view on SV recycling modes (Watanabe 2016; Watanabe *et al.* 2018; Watanabe *et al.* 2013b). Recently flash-freeze has been optimised for use in acute slices (Borges-Merjane *et al.* 2020; Imig *et al.* 2020) which will permit millisecond timescale high resolution images of synaptic activity in intact circuits from larger animal models.

All of these EM methods, despite the millisecond timescales possible, are preserving a moment in time and thus following the events through time in the same synapse are not possible. Fluorescence

imaging techniques, however, do permit visualisation of dynamic cellular events and processes, although generally do not have the level of spatial resolution or structural information.

2.2 Functional readout of presynaptic activity: exogenous markers

Uptake of fluorescent cargo have long been used as a straight forward method to monitor the extent of endocytosis occurring in cells including neurons. The cargo protein, transferrin, coupled to a fluorophore is commonly utilised for this. Cells are incubated on ice to bind the transferrin ligand and then allowed to internalise the ligand at physiological temperatures (Kirchhausen *et al.* 2008). The drawback of using transferrin internalisation as a marker of endocytosis is that it is generally accepted internalisation only occurs via receptor-mediated endocytosis and may not be subject to the same regulation as SV endocytosis.

Fluorescently-coupled synaptotagmin antibodies can be used to monitor SV retrieval. Synaptotagmin is an integral SV protein and thus following exocytosis has domains which are exposed on the plasma membrane surface. Antibodies against this domain can therefore bind to the exocytosed synaptotagmin and are internalised along with the synaptotagmin molecules during endocytosis. Often synaptotagmin antibodies are incubated with the neurons for an extended period to allow uptake via spontaneous exocytosis and thus, active, recycling synapses can be labelled (Kraszewski *et al.* 1995). The disadvantage of this scheme is that the uptake could be via different pathways to those activated during evoked exocytosis.

To investigate specific pathways with exogenous markers, they require selective internalisation. For example, the uptake of large (40-70 kDa) fluorescently labelled dextran molecules is used to monitor ADBE (Clayton *et al.* 2009; Cousin & Smillie 2021; Nicholson-Fish *et al.* 2015). These are inert sugar molecules which are incubated with the neurons and act as fluid phase markers in much the same way as HRP. However, these large molecules are more readily concentrated into larger endosome invaginations than small individual SVs and thus dextran uptake reports synapses undergoing ADBE specifically, therefore enabling this process to be investigated in isolation. Depending on the analysis undertaken, fields of neurons can be automatically counted or uptake at individual neurons can be quantified, for example, through transfection of a marker, making this technique applicable to a range of experimental questions (Cousin & Smillie 2021).

When an SV recycling pathway defect is suspected but the location of the defect unknown, it can be advantageous to initially investigate overall membrane cycling without targeting specific pathways. Use of generic fluorescent membrane markers also negate the requirement to transfect an exogenous reporter. One such example is the family of FM dyes. These fluorescent amphipathic molecules have a variable length hydrophobic lipid tail which determines membrane affinity and a charged hydrophilic head group, ensuring the molecule remains in the outer leaflet of the plasma membrane. The double bond structure determines the spectral properties with green (FM1-43, FM2-10) and red-shifted variants (FM4-64) (Betz *et al.* 1996; Ryan 2001). The dyes are highly fluorescent when integrated into a lipophilic environment but exhibit limited fluorescence in an aqueous environment, thus uptake of the FM dyes results in a measurable increase in fluorescence (Henkel *et al.* 1996). This approach is frequently used as an endpoint assay to investigate the extent of endocytosis (examples include (Betz *et al.* 1992; Matta *et al.* 2012; Yao *et al.* 2010) but the FM dyes are also ideal for monitoring SV recycling in real-time providing data on kinetics as well as multiple stages of the SV cycle (examples include (Ryan 1999; Cheung *et al.* 2010; Vanden Berghe & Klingauf 2006). FM dyes are incubated with the neurons during and just after stimulation facilitating

labelling of any internalising membrane. Following a rest period the labelled membrane can be re-released, reflecting the recycling of SVs. Thus, the extent of endocytosis can be quantified (Cousin *et al.* 2018). Although this technique is informative, because the amount of dye internalised is dependent on the previous exocytic step, an exocytosis defect could confound the results and thus it is important to determine if there is an exocytic defect. This can be achieved by monitoring the kinetics of dye release as a proxy for exocytosis (Cousin *et al.* 2018) or by using a different independent technique.

Recently two new styryl probes have been engineered to have greater photostability and brightness. SP-468 (similar absorption and emission spectra to FM1-42) and SQ-535 (red-shifted variant) were engineered from FM1-43 but in contrast to FM1-43, these dyes associate with the outer leaflet of the bilayer rather than inserting into the bilayer (Collot *et al.* 2020). Although proven to stain the plasma membrane in a range of cell types, they remain untested in terms of vesicle recycling, however, the properties of the dyes may make them a useful more photostable alternative to FM dyes.

FM dyes are also available in a lysine modified version facilitating use with aldehyde fixatives, but these are often fixed inefficiently and are washed out (Revelo *et al.* 2014). Alternatives for a fixed end point assay include membrane-binding fluorophore-cysteine-lysine-palmitoyl group (mCling, (Revelo *et al.* 2014)) or one of the MemBright markers (Collot *et al.* 2019). The mCling label is based on a polypeptide coupled to a membrane anchor and a range of fluorophores allowing flexibility depending on any other fluorophores present in an experiment. The mCling molecules are efficiently fixed and remain bound to the labelled organelles through permeabilisation and immunostaining and have thus been used to effectively label membrane-recycling intermediates (Revelo *et al.* 2014) and investigate endocytosis (Gowrisankaran *et al.* 2020).

The MemBright markers are based on cyanine fluorescent molecules coupled to zwitterionic anchors. They emit in the orange to the near infrared wavelength making them compatible with dual imaging of other fluorescent labels. The MemBright markers when in aqueous solutions form non-fluorescent soluble aggregates, however, in the presence of lipids readily dissociate in the membrane, dequenching the fluorescence (Collot *et al.* 2019). This is analogous to the FM dyes but in contrast to mCling, which is fluorescent regardless of the environment. The MemBright markers are incredibly bright and stably fluorescent for long periods of time making them well adapted to follow intracellular events in live-cell assays. In addition to conventional microscopy, they are also well adapted to super-resolution microscopy (Collot *et al.* 2019) and thus have great potential for multi-channel high resolution imaging.

High resolution imaging is also possible by photo-converting FM dyes thereby allowing visualisation of structures labelled with FM dyes by electron microscopy (Harata *et al.* 2001; Opazo & Rizzoli 2010; Schikorski 2010). Neurons are loaded with FM dyes and following fixation are exposed to high energy blue light in the presence of DAB to generate a precipitate visible by EM. This technique has been used effectively to examine which vesicles are recycled and facilitate tracking of internalised membrane (Welzel *et al.* 2011) as well as to estimate the probability of release in cultured neurons (Branco *et al.* 2010). Additionally, this technique has been optimised for use in acute slices and thus can be used to correlate function with ultrastructure in an intact circuit (Marra *et al.* 2014; Dobson *et al.* 2019).

Differential fluorescence, such as that of the FM and MemBright dyes, is an effective tool to monitor SV recycling. There are also pH-sensitive dyes, such as Cypher5 (Briggs *et al.* 2000) and pHrodo (Chen *et al.* 2013), which can be utilised to monitor presynaptic processes. CypHer, like the

MemBright dyes, is based on a cyanine molecule although in this case it is modified such that it has a pKa of 6.1 (Briggs *et al.* 2000), rendering the dye almost non-fluorescent at pH7.4 but with a peak fluorescence at less than pH5 (Adie *et al.* 2002), making it ideal to image internalisation events into acidic compartments like endocytic intermediates and SVs. pHrodo works on a similar principle and was originally a pH-sensitive version of rhodamine (Chen *et al.* 2013), although it is additionally available as a green fluorescent version. Both cypher and pHrodo dyes can be chemically coupled to other molecules allowing production of bespoke reporters, for example coupling of cypHer to phospholipids allows integration into the plasma membrane and internalisation in much the same way as FM dyes, with the advantage that fluorescence increases the more acidic the compartment becomes (Kahms & Klingauf 2018). CypHer can also be covalently linked to antibodies in order to report internalisation of specific reporters such as synaptotagmin, for example (Henkel *et al.* 2019). CypHer and pHrodo can also be coupled to generic molecules like dextran, creating a fluid-phase marker which can be used to mark endocytic compartments (for an example using pHrodo-dextran, see (Harper *et al.* 2016)).

2.3 Functional readout of presynaptic activity: Genetically encoded reporters

Many of the previously discussed techniques to monitor the SV lifecycle are dependent on internalisation or uptake of markers, making dissection of exocytosis from endocytosis difficult. By fusing reporter proteins to the luminal domain of a SV protein, it is possible to monitor SV fusion and subsequent retrieval, resolving this limitation. Most commonly a version of green fluorescent protein, modified to be exquisitely pH sensitive, pHluorin, is used. The original pHluorin construct, synaptophluorin, has the pHluorin molecule fused to synaptobrevin2 (Miesenböck *et al.* 1998), although a range of SV proteins including, but not limited to, synaptophysin (Granseth *et al.* 2006), synaptotagmin (Fernández-Alfonso *et al.* 2006), and vesicular glutamate transporter 1 (vGlut1, (Voglmaier *et al.* 2006)) are commonly used.

They operate on the principle that in an acidic environment the fluorescence of the fluorophore is quenched and when in a more neutral environment this quenching is removed and the proteins are fluorescent. Thus, when the SVs incorporating the reporter fuse with the plasma membrane during exocytosis, this is visualised as an increase in fluorescence and endocytosis is represented as a decrease in fluorescence as the reporters are retrieved from the plasma membrane (Granseth *et al.* 2006). These measurements provide an estimate of the extent of exocytosis and endocytosis as well as kinetic information on these processes. Manipulation of the extracellular buffer to contain ammonium chloride also allows calculation of the size of the SV pool which fuse as a proportion of the total SV pool. The ammonium chloride collapses the intracellular pH gradients and reveals the entire pHluorin labelled SV pool (Sankaranarayanan & Ryan 2000). Further, washing with an impermeant acidic solution quenches any plasma membrane fluorescence enabling calculation of the steady state surface pool as well as detection of any surface stranding caused by a block in endocytosis (Sankaranarayanan & Ryan 2000).

The range of aspects of presynaptic function that can be monitored using pHluorins makes them an ideal choice for an investigative tool to screen a range of presynaptic factors which could be disrupted in disease. Indeed our lab and others have utilised pHluorins to identify presynaptic defects in Huntington's disease (slowing of clathrin-mediated endocytosis; (McAdam *et al.* 2020), neurodevelopmental disorders (slowed kinetics of exocytosis; (Baker *et al.* 2018); efficient retrieval of SV proteins; (Gordon & Cousin 2013)), epilepsy (defects in activity-dependent trafficking of synaptotagmin;(Harper *et al.* 2020)), Parkinson's disease (disrupted SV clustering; (Nemani *et al.*

2010)) and Alzheimer's Disease (multiple stages of SV lifecycle impacted; (Park *et al.* 2013; Zhou *et al.* 2017; Lazarevic *et al.* 2017) to highlight a few examples.

However, pHluorins are not without their limitations and it should be born in mind that the decrease observed upon endocytosis also contains a component of SV reacidification. This process is however much faster than endocytosis and so is not likely rate-limiting (Sankaranarayanan & Ryan 2000). Reacidification can be investigated using timed-acid buffer incubations in order to determine if there the condition under test impacts reacidification (Sankaranarayanan & Ryan 2000; Soykan *et al.* 2017).

Further, the fluorescent increase seen upon exocytosis is representative of the net flux of vesicles and therefore also contains of an endocytic component. This can be dissected however, by conducting experiments in the presence of a proton pump inhibitor like bafilomycin or folimycin (Sankaranarayanan & Ryan 2000). This prevents the reacidification of SVs and thus quenching of pHluorin molecules once they have been exocytosed, allowing exocytosis to be assayed in isolation. By comparing the peak in the absence and presence of bafilomycin, the extent of endocytosis occurring during exocytosis can also be estimated (Kim & Ryan 2009).

Although pHluorins are used to monitor exocytosis and endocytosis at the process level, the data generated, is strictly speaking, information about the recycling of the tagged SV protein and not simply SVs in general. This can be used as an advantage however, and allows investigation of the function and retrieval of individual proteins. In more general terms, this limitation can be mitigated by the use of several pHluorin constructs which would delineate between a general process defect and one specific to an individual protein. The use of red-shifted pH sensitive molecules such as mOrange, or the improved mOrange2, in combination with pHluorins also opens up the possibility of tracking multiple SV proteins at the same time allowing comparison of trafficking of different cargo simultaneously (Shaner *et al.* 2008; Raingo *et al.* 2012; Harper *et al.* 2017).

Other red-shifted variants such as mCherry(I158E/Q160A) have also been designed to respond to pH in ratiometric manner. These sensors, although possibly having a lower dynamic range, are unaffected by differences in the extent of reporter expression and the effects of photobleaching (Rajendran *et al.* 2018). They can also be multiplexed with other fluorophores marking different compartments and increasing their utility.

Use of pH sensitive moieties has traditionally been used to investigate SV exo-/endocytosis but the range of molecules tagged is expanding to address other questions. For example, trafficking of many of pHluorin-tagged SV proteins proceeds predominantly via clathrin-mediated endocytosis but by fusing the pHluorin onto cargo specific for other retrieval pathways, these can be examined. For example VAMP4 is specific to ADBE and thus VAMP4-pHluorin has been used to investigate the factors regulating this pathway (Nicholson-Fish *et al.* 2015). The role of different isoforms of the vGLUT channels has been addressed by tagging each isoform (Li *et al.* 2017) and secretory peptides have been tagged in order to study their regulated release (see Brain Derived Neurotrophic Factor-pHluorin (Yu *et al.* 2018) or Neuropeptide Y-pHluorin (Makhmutova *et al.* 2017; Wang *et al.* 2017b)). By comparing vGLUT-pHluorin recycling with that of vGAT-pHluorin recycling, differences in pH dynamics and vesicle filling have been elucidated in excitatory versus inhibitory synapses (Herman *et al.* 2018). Most recently this approach has been refined so that the pHluorin-tagged proteins were expressed under the control of excitatory and inhibitory synapse promotors (Ca2+/calmodulin-dependent kinase II alpha and GAD67, respectively) in order to better control over-expression of the reporters in the specific sub-set of neurons (Bae *et al.* 2020). pHluorins are also being used in acute slices (Park 2018) and mice have been genetically modified to express pHluorin in subsets of neurons

(Li *et al.* 2005; Araki *et al.* 2005), further expanding the utility of this technique to examine presynaptic function.

PHluorin imaging in principle could be useful for investigating any acidic organelle and indeed lysosomal trafficking has also been investigated using pHluorins (Golan *et al.* 2019). However new genetically encoded reporters which increase in fluorescence in an acidic environment have been engineered (Shinoda *et al.* 2018). In order to achieve this, acid brightening Fluorescent Protein (abFP) consists of a positively charged GFP-based chromophore generated by incorporation of an artificial quinolone-containing amino acid, Qui. In an acidic environment Qui is protonated, resulting in fluorescence. abFP has been used to follow receptor endocytosis (Fu *et al.* 2018) and may be of value for investigating the endosomal pathways. However, use of this probe is reliant on the ability to generate a corresponding unique tRNA in the system, limiting the use beyond easily genetically manipulatable cell lines. A more accessible probe to monitor pH dynamics throughout the acidic compartments in a cell is the ratiometric reporter pH-Lemon. pH-lemon is based on a pair of fluorescent proteins, mTurquiose2 and EYFP, fused together via a linker region. The pH of the environment controls the fluorescence of the EYFP and the efficiency of the Forster resonance energy transfer (FRET, for detailed information on the principles of FRET, see the following section) between the proteins, allowing the pH throughout the cell area being illuminated to be sensitively measured. Using this technique, pools of neutral and acidic vesicles in the endolysosomal system have been identified (Burgstaller *et al.* 2019) and it could be applied to look at reacidification and SV/endosomal trafficking following exocytosis.

Multiplexing with different fluorophores allows interrogation of multiple proteins at once. The same principle can be applied to multiplexing of different sensors to interrogate more than one function simultaneously. This approach has been used successfully with pHluorin and the calcium sensor, GCaMP4 (Jackson & Burrone 2016) as well as mOrange2-vGLUT and synaptophysin-GCAMP3 (Li *et al.* 2011) to simultaneously monitor SV recycling and calcium influx. There is scope to extend the multiplexing approach, depending on the fluorophore excitation/emission profiles, to include a range of sensors including genetically encoded voltage sensors to monitor membrane depolarisation (Kang *et al.* 2019) as well as ligand binding reporters to monitor the neurotransmitter released (O'Banion & Yasuda 2020). The latter is particularly interesting as multiplexing a sensor examining SV recycling with one which reported the output of SV release may be informative.

The intensity-based fluorescent reporter (iSnFR) family are based on a ligand binding domain fused to a version of GFP. The conformational change elicited when the ligand is bound, facilitates the fluorescence of the fluorophore. The most commonly used version is iGluSnFR, which detects glutamate release although the family also consists of other variants which can detect GABA, ATP and glucose (O'Banion & Yasuda 2020). There are also variants of iGluSnFR which can respond to and resolve high frequency stimulations (Helassa *et al.* 2018) as well as others containing different fluorophores to generate blue, cyan and yellow variants (Marvin *et al.* 2018), facilitating flexibility to utilise different combinations of reporters.

Additionally there are genetically encoded sensors based on G-protein couple receptors which are designed to detect other neurotransmitters such as dopamine (G-protein coupled receptor activation based dopamine ($GRAB_{DA}$, (Bae *et al.* 2020; Sun *et al.* 2018) and dLight, (Patriarchi *et al.* 2018)) and acetylcholine (GACh, (Jing *et al.* 2018) and $GRAB_{ACh}$, (Jing *et al.* 2020)). These sensors are based on the dopamine and muscarinic receptors, respectively, with a fluorescent protein engineered into an intracellular loop. Upon binding the appropriate neurotransmitter, the conformational change is transduced to the fluorescent protein facilitating a large increase in fluorescence. The ability to accurately monitor the release of neurotransmitter concurrently with

the ability to monitor components of the SV life cycle have the potential to correlate events occurring on both sides of the synapse, providing valuable insight into synaptic function. Although phluorin-based reporters are lagging behind neurotransmitter sensors in terms of *in vivo* applications, this represents a clear avenue for future research.

3. Interrogation of the molecular mechanism underlying an aberrant phenotype

The techniques discussed above are, in general, focussing on the (dys)function of the synapse. Even when the genetic mutation for a condition or model is known, often the interactions at the protein level responsible for the phenotypes are not. A common approach, therefore, is to investigate the protein networks, interactions and any alterations to post-translational modification which may be responsible. This information in turn, can be fed back into the approaches used above to evaluate whether altering the identified target can modulate the aberrant phenotype. This can be achieved using a non-biased approach by using the whole cell or tissues as input or in a more targeted fashion by isolating interacting partners of specific proteins for example.

3.1 Identification of molecular targets

In recent times there has been an explosion in the information generated from so called "-omics" studies. Not only has this approached identified new targets for study but has also provided insights into the composition and regulation of the synapse. This has been particularly valuable for investigation of proteins with links to disease. Comparative proteomics has been extensively used to determine differences in the protein networks and interaction partners in disease (for some illustrative examples see Alzheimer's Disease (Mendonça *et al.* 2019; Chang *et al.* 2014; Krivinko *et al.* 2018; Hesse *et al.* 2019; Wang *et al.* 2017a), Huntington's Disease (Vodicka *et al.* 2015; Skotte *et al.* 2018), Parkinson's Disease (Connor-Robson *et al.* 2019), Neuronal Ceroid Lipofuscinosis (Llavero Hurtado *et al.* 2017), Amyotrophic lateral sclerosis (Engelen-Lee *et al.* 2017), prion diseases (Peggion *et al.* 2019; Gawinecka *et al.* 2013), ageing (Graham *et al.* 2019) and also neurodevelopment (Broek *et al.* 2016)).

With improvements in sensitivity and sample preparation methods, dynamic changes in post-translational modifications are also detectable. For example phosphorylation mapping on a large scale to determine phosphorylation changes in synaptic proteins in response to stimulation and activity (Engholm-Keller *et al.* 2019) or alterations in glycosylation (Fourneau *et al.* 2020; Sytnyk *et al.* 2020) and palmitoylation (Zareba-Koziol *et al.* 2019) have been conducted. Diversifying these methods has the potential to identify other dynamic post-translational modifications important for the regulation of presynaptic function and plasticity.

Measuring protein turnover and half-life via Stable Isotope Labelling by Amino acids in Cell culture (SILAC) in model systems versus controls can be used to pinpoint alterations to individual targets or pathways and thus provide valuable insight into pathophysiological mechanisms (Cohen & Ziv 2019). Cultured cells are incubated with heavy labelled versions of amino acids which in turn are incorporated into synthesised proteins (Ong *et al.* 2002). Detection of the peptides at time intervals following incubation allows the estimation of protein half-lives or a measure of protein turnover. This approach has been used to characterise the protein half lives in different primary cell cultures (Mathieson *et al.* 2018) and more specifically to compare the half lives of proteins in neurons versus glial cells (Dörrbaum *et al.* 2018). SILAC has also been used to measure the differences in

degradation in response to inhibition of the proteasome, illustrating that half life estimations are valuable factors in determining cellular response to a pharmacological insult (Hakim *et al.* 2016) but could also hold real value following genetic manipulation such as disease models. This would be especially powerful in combination with Stable Isotope Labelling in Mammals (SILAM) whereby the labels are introduced through the diet of the animals enabling quantification of turnover in an *in vivo* context (Cohen & Ziv 2019) – this has been utilised to determine changes in protein lifetime following plasticity alterations (Heo *et al.* 2018) but could perhaps also be used to map alterations through disease progression.

A limitation to SILAC is that it is dependent upon the use of cultured cells and although SILAM does provide an alternative *in vivo* context for labelling, interpretations are complicated by the metabolic pathways in the whole animal (Cohen & Ziv 2019). Bowling *et al* (Bowling *et al.* 2016) combined SILAC with Bioorthogonal noncanonical amino acid tagging (BONCAT) to generate a new technique, BONLAC, which they were able to use in brain slices. This technique allows the determination of protein turnover in intact slices thus going some way to preserving the higher circuit structure found *in vivo* but at the same time allowing manipulation of the conditions by either pharmacological means or by electrical/optical stimulation.

3.2 Identification of protein interactions

Proteomics are also key to identifying potential interactors in more targeted approaches. Mass spectrometry is extensively used to determine the identity of protein interactions present at the synapse. Immunoprecipitatons and pulldown assays are often used to identify interactors as well as to provide strong evidence that protein partners interact. These can be powerful approaches to interrogate a system, however, the nature of these techniques require cell lysates as input material. This means that the environment in which the proteins may interact is disrupted and the efficiency is dependent on the affinity and specificity of the bait, which is the antibody or fusion protein in question.

Understanding the interactions between key proteins within the presynaptic nerve terminal is crucial to gaining a complete understanding of the molecular mechanisms underpinning synaptic vesicle exocytosis and recycling. This is particularly true for protein-protein interactions that change upon stimulation, or, for example, SNARE proteins that undergo a sequence of steps and interactions to ensure priming, docking and fusion of SVs. As such, being able to measure protein-protein interactions *in situ* and in real time is key to furthering our understanding of these processes. One such method that is useful for this purpose is FRET.

In FRET imaging, proteins of interest are typically labelled with two fluorescent molecules, either a dye or, more commonly, a fluorescent protein. There needs to be sufficient spectral overlap between these two proteins. One is considered the donor and the other the acceptor. FRET occurs if the two fluorescent proteins are within approximately 10 nm during which, following excitation of the donor, dipole-dipole transfer of energy to the acceptor occurs (Cui *et al.* 2019). This results in the quenching of the donor fluorescence and therefore can be measured, providing an indirect measurement of the proximity of the two proteins.

Little intervention is required for FRET imaging and it can be carried out in living cells, or even *in vivo*, enabling precise temporal and spatial measurements of protein interactions. However, this technique does require careful experimental planning and control experiments to ensure that data is correctly interpreted. These requirements have recently been detailed in Nature Methods with a

detailed methodological analysis of the use of FRET, where the authors argue for a standardised method of undertaking and analysing FRET experiments to ensure accurate interpretation and reproducibility (Algar *et al.* 2019).

Perhaps the most common method to calculate FRET is by the measurement of the changes in fluorescence of both the donor and acceptor, however acceptor photobleaching can also be used. Through this method, the difference in donor fluorescence is measured before and after photobleaching of the acceptor fluorescent protein. If FRET is occurring then the fluorescence is expected to increase following removal/bleaching of the acceptor.

The most reliable method for measuring FRET is through Fluorescence Lifetime Imaging Microscopy (FLIM). The energy transfer between two closely associated molecules results in an increase in the rate of exponential decay of fluorescence following excitation, which can be measured. FRET-FLIM was used to examine the interaction between two SV proteins, Synaptophysin and Synaptobrevin-II, and showed that their interaction was lost upon stimulation of the cultured neurons with latrotoxin (Pennuto *et al.* 2002), suggesting disassembly of their interaction is required for fusion of SVs.

One limitation is that the FRET measurement includes a heterogeneous population of proteins within a cell that can be in a combination of different states and as such may not detect small changes to the interactions of proteins. However, single molecule FRET (smFRET) can be carried out. While this technique is usually carried out on proteins *in vitro* that are diffusing in solution, FRET has recently been combined with single molecule imaging in cultured cells to measure the interactions of individual molecules during single particle tracking. This method was used to measure membrane proteins (Sakon & Weninger 2010), or more recently using alternative laser excitation to measure cytosolic proteins (Okamoto *et al.* 2020). Combining FRET with recent advances in microscopy enables continual development of our knowledge of synaptic proteins, and one set of proteins in particular - the SNARE proteins. FRET, smFRET and FRET-FLIM have all been used extensively to aid in the understanding of the steps leading to fusion of SVs upon stimulation (Verboogen *et al.* 2017; Takahashi *et al.* 2015). Additionally, FRET has also been combined with a liposome fusion assay to examine SNARE complex assembly and the factors which regulate fusion (Prinslow *et al.* 2019; Stepien *et al.* 2019; Weber *et al.* 1998; Martens *et al.* 2007; Schaub *et al.* 2006).

In addition to smFRET, combining FRET–FLIM with two-photon imaging has enabled imaging in slice culture preparations (Takahashi *et al.* 2015) and even the brain of living mice (Matsuda & Terai 2020) providing wide scope to further research *in vivo* and at the single molecule level. Given the recent developments in advanced microscopy techniques, there is much potential to combine these with FRET to enhance our understanding of protein-protein interactions within the presynaptic nerve terminal.

3.3 Identification of target protein localisation

Identification of which proteins/lipids interact is critical to determine molecular mechanism, however, this is of limited value without an understanding of where in a cell these interactions take place and in which cellular context. To investigate this, techniques with high spatial resolution are required to combine molecular detail with the cellular environment. Electron microscopy is often the gold standard for localisation but this is not particularly high throughput and requires fixed time point samples. The huge advances in imaging technology combined with computer image processing, however, mean that super-resolution microscopy is almost able to provide the spatial resolution of electron microscopy but also dynamic temporal information. Super-resolution

microscopy, however, is limited in the extent of the ultrastructural context it can provide and is also dependent on specialised microscopes and molecular tools. Both are discussed in the following section and ultimately the best approach will depend on the research question.

3.3.1 Immuno-Electron microscopy

Combined with immuno-staining, electron microscopy is capable of resolving protein localisation to nanometer resolution. Determining protein localisation using EM has the advantage that localisation is in context relative to the cellular ultrastructure. Traditionally, proteins are localised via antibodies coupled to a detection system, often heavy metal particles such as gold of defined radius (Hacker & Lucocq 2014). These particles are then detected in the electron beam. Rather than gold particles, antibodies can be coupled to catalytic domains such as horse-radish peroxidase (HRP) which generate an electron dense diaminobenzidine (DAB) product in the presence of hydrogen peroxide. This is effective but is limited by the specificity and labelling of the antibodies and often requires permeabilisation of the cells/tissues in order for the antibodies to access the antigens (Loussert Fonta & Humbel 2015; Ellisman *et al.* 2012; Griffiths & Lucocq 2014).

These disadvantages have been tackled in recent years with the generation of genetically encoded EM tags (Martell *et al.* 2017) whereby the detection system is directly coupled to the protein of interest as a fusion protein. This can then be transfected or transduced into the cellular system/tissue analogous to a fluorescent protein conjugate. This process produces a localised electron dense signal which is quantifiable. HRP has been used in this manner but is not effective as a label in all cellular compartments due to the inability of the secondary structure to form correctly out of the reducing environment of the secretory pathway (Martell *et al.* 2012). Ascorbate peroxidase (Martell *et al.* 2012) and the enhanced version, APEX2 (Martell *et al.* 2017) provide an alternative genetically encoded-EM tag which is functional in all cellular environments. It is based on a naturally occurring plant peroxidase and in a similar manner to HRP, is able to generate a highly localised DAB precipitate in the presence of hydrogen peroxide (Martell *et al.* 2012).

Other genetically encoded-tags have proved useful for correlated light and EM, allowing the detection of fluorescence in live cells prior to fixation and processing for EM to determine the ultrastructure (Martell *et al.* 2017). One such example is mini Singlet Oxygen Generator (miniSOG) originally engineered by the Tsien laboratory (Shu *et al.* 2011). MiniSOG is a fusion protein where the moiety fused to the protein of interest is intrinsically fluorescent and able to catalyse the generation of reactive oxygen species when illuminated with blue light in the presence of oxygen gas. This is turn facilitates the production of the DAB precipitate (Shu *et al.* 2011). Although powerful, this technique does require specialised equipment and only allows one field of view to be fixed at a time.

Most recently ferri-tag has been developed by the Royle laboratory to overcome the often low resolution generated by diffuse DAB staining (Morphew *et al.* 2015). Ferri-tag is based on the ability of FK506 binding protein (FKBP) to dimerise with the FKBP-rapamycin binding (FRB) domain of mammalian target of rapamycin (mTOR), in the presence of rapamycin. The protein of interest is fused to the FKBP fragment and the ferritin-tag is engineered onto the FRB fragment. Both are introduced into cells and in the presence of rapamycin, create a specific ferritin localised signal (Clarke & Royle 2018). In addition, both molecules have a fluorescent marker and so this technique can also be used for correlated EM. The disadvantage of this system is the reliance on multiple constructs which require transfection at the same time. This will not likely be limiting when comparing protein localisation between wild type and disease model for example but could prove

more technically challenging if other factors to manipulate the expression level of a specific protein also require transfection.

3.3.2 Super-Resolution microscopy

One of the main restrictions to understanding the function of the presynaptic nerve terminal using fluorescence microscopy has been its size. Small central nerve terminals are approximately 1-2um in width, which means that due to the diffraction limit of conventional microscopy, sub-cellular structures and more specifically, the trafficking of synaptic vesicles cannot be resolved. The diffraction limit is due to the light waves forming a point spread function, which is approximately half the width of the wavelength of the emitted light (~250 nm). Therefore, any objects that are closer together than the size of the point spread function cannot be resolved (Galbraith & Galbraith 2011).

One field that has gained popularity due to recent advancements is super-resolution microscopy. Super resolution microscopy refers to a variety of techniques that have overcome the diffraction limit using different methods. The benefits and limitations of these depend on the technique but often result in a resolution between 10 and 100 nm, greatly increasing the ability to visualise small sub-cellular structures (Reshetniak & Rizzoli 2019). The techniques can be split broadly into three categories (Figure 2): single molecule localisation microscopy, Stimulated emission depletion microscopy (STED) and expansion microscopy depending whether the increased resolution is gained by manipulating the molecules being imaged, the beam exciting the fluorophores or the preparation of the sample, respectively.

3.3.2.1 Single Molecule Localisation Microscopy

Single molecule localisation microscopy encompasses several techniques that all rely on the ability to image and localise single fluorescent molecules. Fluorescent molecules are sparsely 'switched on and off', through different methods described below, enabling visualisation of the point spread function. Software is then used to calculate the center of the point spread function, pinpointing the precise location of the molecule. Through imaging over extended periods of time the location of individual molecules are accumulated, enabling the reconstruction of a high-resolution image (Betzig *et al.* 2006; Rust *et al.* 2006; van de Linde *et al.* 2011; Reshetniak & Rizzoli 2019). Alternatively, in living cells, this same method can be applied to single particle tracking whereby the movement of a molecule can be tracked over time at very high resolutions (Dupuis & Groc 2020). These are extremely powerful techniques as they provide resolutions of up to 10 nm in XY and can also be applied to 3D samples (Zhong 2015).

Discussed below are examples of approaches to single molecule microscopy:

1) *Direct Stochastic optical reconstruction microscopy (dSTORM)*

This commonly used technique enables the acquisition of individual molecules through the quenching and stochastic recovery of organic fluorophores (Heilemann *et al.* 2008; Zhong 2015). dSTORM is capable with the use of conventional organic fluorophores, such as Alexa Fluor 647 and Cy5, and as such can typically be applied to immunostained samples (Heilemann *et al.* 2008). It has been widely used to examine the architecture of the synapse in cultured neurons and has provided high-resolution 3D maps of multiple synaptic proteins relative to the active zone and post-synaptic density (Sigal *et al.* 2018; Dani *et al.* 2010; Klevanski *et al.* 2020). Additionally, this technique has been tested in brain slices, enabling high-resolution, 3D imaging of synaptic vesicles from *in vivo* samples (German *et al.* 2017; Lehmann *et al.* 2015).

Whilst dSTORM provides powerful, high-resolution information on the architecture of the synapse, it can typically only be used with fixed samples and therefore the ability to gain functional information is limited. Combining dSTORM with electron microscopy provides information on the highly-resolved localisation of a particular protein within the ultrastructure of the synapse (Mateos *et al.* 2018; Persoon *et al.* 2018). Correlating dSTORM with functional SV recycling assays has also been highly beneficial and has revealed different subpopulations of synaptic vesicles (Harper *et al.* 2016).

2) Photo-activated localisation microscopy (PALM)

PALM is carried out by using photo-activatable or photo-convertible fluorescent proteins, such as mEos2, which are expressed with a protein of interest. Stochastic activation of these fluorescent proteins is achieved using low levels of illumination and results in the ability to distinguish and localise individual molecules (Betzig *et al.* 2006; Zhong 2015).

PALM, in combination with dSTORM, has been used extensively to understand the role of the plasma membrane SNARE protein, Syntaxin-1, in synaptic vesicle exocytosis (Padmanabhan *et al.* 2020). Both techniques have been used to characterise clusters of syntaxin-1 in neurosecretory cells and cultured neurons (Bar-On *et al.* 2012; Pertsinidis *et al.* 2013; Kavanagh *et al.* 2014; Smyth *et al.* 2013; Kasula *et al.* 2016). Recently *Drosophila melanogaster* have also been generated that express syntaxin1A-mEOS2, enabling SPT-PALM at motor nerve terminals *in vivo* (Bademosi *et al.* 2017; Bademosi *et al.* 2018). Overall these works have led to a greater understanding of Syntaxin-1A, its interactions and how these contribute to regulating exocytosis (Padmanabhan *et al.* 2020).

3) universal Point Accumulation Imaging in Nanoscale Topography (uPAINT)

This technique has been hugely beneficial to the understanding of the diffusion of membrane proteins in living cells. Nanobodies targeting the extracellular region of a protein of interest are labeled with fluorescent dyes that are added during image acquisition (Giannone *et al.* 2010; Giannone *et al.* 2013). As low concentrations of the nanobodies are used, this enables the visualisation of single molecules and the tracking of these proteins on the plasma membrane. One of the benefits to this technique is that the trafficking of endogenous proteins can be examined, however uPAINT can also be applied to proteins expressing an extracellular tag. This technique has been used extensively to understand the diffusion of AMPA receptors at the post-synaptic terminal (Choquet & Hosy 2020) and has also been applied to the presynaptic plasma membrane SNARE, syntaxin1A (Kasula *et al.* 2016). Recent work has examined the trafficking of SV proteins that are specifically located on the plasma membrane. uPAINT was used in combination with pHluorin assays to identify mislocalisation of an epilepsy-associated mutant of SV2A to the plasma membrane and correlated this with altered membrane diffusion, providing a potential underlying cause for the disorder (Harper *et al.* 2020).

4) Single particle tracking of Synaptic Vesicles

uPAINT has also been adapted to track the mobility of internalized synaptic vesicles, termed subdiffractional Tracking of Internalised Molecules (sdTIM) (Joensuu *et al.* 2017; Joensuu *et al.* 2016). With this technique nanobodies bind to synaptic vesicle proteins and are internalized during an incubation period prior to imaging. Therefore, only endocytosed SV proteins are visualised. This study found that SVs underwent different forms of movement indicating different states (Joensuu *et al.* 2017; Joensuu *et al.* 2016). This technique can also be applied to tracking other organelles, such as retrograde carriers and endosomes (Wang *et al.* 2016; Joensuu *et al.* 2017).

Other groups have also developed methods to measure the movement of SVs. One study used the lipophilic dye, SGC5, to label single SVs in nerve terminals that are then localized and tracked (Forte *et al.* 2017). This group also found that SVs underwent different movements (Forte *et al.* 2017). The development of novel membrane dyes such as Membright and mCling provide further opportunities to develop this technique (Collot *et al.* 2019; Revelo *et al.* 2014). However, while the benefit of using a lipophilic dye means that the technique is not reliant on measurements from a specific protein it is currently limited by the fact that only a single SV can be measured in each nerve terminal.

Another group has used sparsely photo-activated RFP tagged to vGLUT in ribbon synapses of retinal bipolar neurons of zebrafish (Vaithianathan *et al.* 2016), however focused on examining the exocytosis of SVs. Whether this technique could be adapted to also examine endocytosis would be of interest.

Overall, significant progress has been made towards developing super-resolution imaging methods that enable the trafficking of SVs within the presynaptic nerve terminal to be examined. However this work is still in the early stages and applying these techniques to key questions regarding the SV recycling pathway will greatly enhance our understanding of the precise molecular mechanisms involved both in control as well as disease models.

3.3.2.2 Stimulated emission depletion microscopy

Stimulated emission depletion (STED) microscopy was one of the first super-resolution techniques to gain prominence (Hell 2007; Hell & Wichmann 1994). STED microscopy functions by using a donut shaped depletion beam in combination with the excitation light. This depletion beam quenches the fluorophores on the edges of the excitation region, reducing the point spread function and thereby increasing the resolution. Indeed, up to 40 nm lateral resolution has been achieved. A key advantage to STED is that imaging is relatively straightforward and specific processing of samples is not required. It can also be carried out on living samples and has been used on brain slices and in the brain of a living mouse (Wegner *et al.* 2018; Masch *et al.* 2018). Additionally, developments in optical clearing methods, particularly cleared lipid-extracted acryl-hybridized rigid immunostaining/in situ hybridization-compatible tissue hydrogel (CLARITY), has also greatly enhanced the opportunities for the imaging of thick tissue and whole brain samples. These clearing methods have recently been comprehensively reviewed (Ueda *et al.* 2020; Parra-Damas & Saura 2020). These techniques have been combined with STED (Ke *et al.* 2016; Hruska *et al.* 2018) and highlight the possibilities for further examining synaptic architecture and circuitry in vivo in the future.

The use of STED has led to several developments in understanding synaptic function, including determining the fate of exocytosed synaptic vesicle proteins (Opazo & Rizzoli 2010; Willig *et al.* 2006) and the dynamics of SV movement (Westphal *et al.* 2008; Kamin *et al.* 2010). Recently, STED has also been used in combination with the membrane dye, mCling, to visualise endocytosed SVs in fixed and living cells (Revelo *et al.* 2014). The application of STED is constantly evolving and has been used in combination with other super-resolution techniques, for example combined with single particle tracking (Inavalli *et al.* 2019), which provides further potential to examine synaptic vesicle recycling pathways in the future.

3.3.2.3 Expansion Microscopy

Expansion microscopy is a relatively novel technique. Rather than manipulating the imaging method, this technique involves embedding samples in a gel that subsequently expands, therefore increasing the volume of the sample (Chen *et al.* 2015). The first experiments carried out using this method

resulted in a resolution of 70 nm, however later work from the same group used an iterative expansion method that gained a resolution of 25 nm (Chang *et al.* 2017). This level of resolution was achieved by another group using an improved gel recipe (Truckenbrodt *et al.* 2018), who notably also applied the technique to brain slices. More recently, expansion microscopy has been used in combination with light sheet microscopy to image large regions of mouse brain or entire drosophila brain (Gao *et al.* 2019; Bürgers *et al.* 2019), indicating the potential of the technique to link synaptic architecture and neural circuits. Light sheet microscopy increases the rate of acquisition and highlights that combining super-resolution with other imaging techniques can have real benefit.

The capability for super-resolution microscopy to contribute to our understanding of synaptic function and synaptic vesicle recycling over the past decade has been hugely beneficial in furthering our knowledge of these systems. However, this is has only been the beginning and there is further potential for these techniques to be combined with other imaging applications to answer key questions on the mechanisms underlying synaptic vesicle recycling.

These different super-resolution techniques each have their own benefits and limitations depending on the desired application, for example, dSTORM provides resolutions of up to 10 nm but is generally limited to fixed samples, whereas the resolution of STED is slightly lower but enables the imaging of live samples, notably the brain of a living mouse (Wegner *et al.* 2018; Masch *et al.* 2018). Super-resolution microscopy has greatly contributed to our understanding of synaptic vesicle recycling and synaptic function and will continue to be of benefit investigating the molecular alterations present in disease.

3.3.3 Correlative EM

Correlative EM can provide the best of both fluorescent imaging and ultrastructural resolution assuming the exact position of a cell can be relocated following sample fixation (Loussert Fonta & Humbel 2015; Ellisman *et al.* 2012). The utility of these techniques is being pushed further still with the combination of super-resolution imaging techniques and EM, allowing the determination of the location of individual protein molecules in a cellular context (Watanabe *et al.* 2014a; Shu *et al.* 2011; Clarke & Royle 2018). Ultimately the choice of method to use will depend on the sample, the tools and the equipment available to image it but there is no doubt that it is possible to identify exactly where a given protein is in a cell and thus when the genetic cause of a disorder is known for example, the localisation or resulting mis-localisation, can be informative.

4. Conclusion and future perspectives

Over the last several decades our understanding of presynaptic function has ballooned however the molecular mechanisms and pathways underpinning the function and dysfunction of the presynaptic nerve terminal remain an active area of research. The potential for this field has expanded with the development of screening assays such as proteomics and the processing of large volumes of data, particularly in regards to the identification of proteins linked to disease. In parallel the application of novel, advanced imaging techniques in combination with well established methods has provided us with the opportunity to further dissect the roles of the proteins in synaptic function. One prime example is the discovery of ultrafast endocytosis and other possible SV endocytosis pathways that until recently had remained unidentified. This review has summarised a range of techniques, each with their advantages and limitations (see Table 1) and ultimately the methods used will depend on the research question being posed. The ability to multiplex different techniques allows more complex questions to be answered and applied to presynaptic function in disease models. A better

understanding of function at the molecular level will in turn lead to the identification of dysfunctional pathways and direct research towards potential new therapeutic targets.

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Conflicts of interest
The authors have no conflicts of interest to declare.

Figure legends

Figure 1 - Summary of the synaptic vesicle pathways at the presynapse. The schematic depicts several of the pathways operational in a presynaptic bouton. Neurotransmitter containing vesicles are filled with neurotransmitter via a proton-motive force. In response to an action potential invading the presynaptic bouton and depolarising the membrane, voltage-sensitive calcium channels open, facilitating calcium influx. This in turn activates either partial fusion of the synaptic vesicles in a process termed 'Kiss and Run' or full collapse of the synaptic vesicle into a region of the plasma membrane, the active zone, releasing neurotransmitter into the synaptic cleft. Collectively this is termed exocytosis. Following vesicle fusion, synaptic vesicles can be reformed by multiple endocytic pathways. Clathrin-mediated endocytosis (CME), retrieves single vesicles at a time and requires the coat protein clathrin (dark blue spheres). Ultrafast endocytosis (UFE) and Activity-Dependent Bulk Endocytosis (ADBE) are illustrative of pathways which retrieve excess membrane to form an endosome which later buds to regenerate synaptic vesicles. ADBE is only activated during high intensity activity whereas ultrafast endocytosis can be operational in response to single action potentials but is only detectable at physiological temperatures.

Figure 2 - Summary of super-resolution imaging techniques. (A,B) Single molecule localisation microscopy relies on the use of algorithms to pinpoint the precise location of fluorescent molecules. To enable visualisation of the individual fluorescent molecules, they are sparsely 'switched on' one after the other over time. Their calculated locations are then used to either build up a detailed image of the protein of interest (A) or to track their movements over time (B). (C) Stimulated emission depletion microscopy uses a donut shaped depletion beam following excitation to quench the excited fluorophores in the outer region, which therefore results in an increased resolution image. (D) Expansion microscopy involves the embedding of samples within an expanding gel. This results in an increase in volume of the sample, which provides greater resolution of the cellular structures during imaging.

Table 1 – Techniques to investigate presynaptic function. Table summarises the advantages and limitations of the techniques discussed in the review.

Table 1 – Techniques to monitor presynaptic function

Technique	Advantages	Limitations	Example references
Electron microscopy	• High spatial resolution • Possible to generate serial reconstructions to form a 3D-rendering	• Low throughput • Fixed point in time • Requires samples to be fixed either with chemical fixatives or flash freezing	(Pechstein *et al.* 2020; Cao *et al.* 2017; Gowrisankaran *et al.* 2020)
− Combined with fluid phase or membrane markers eg. HRP or FM dyes	• Monitor multiple endocytic pathways simultaneously	• Require processing of the marker to be visible by EM	(Clayton *et al.* 2008; Opazo & Rizzoli 2010; Schikorski 2010; Welzel *et al.* 2011; Dobson *et al.* 2019)
− Combined with antibodies against specific proteins	• Identify cellular location of a defined target with nanometer resolution	• Dependent on affinity and specificity of the antibody • Often requires permeabilisation of samples to allow antibodies to access intracellular antigens	(Nicholson-Fish *et al.* 2015; Cottrell *et al.* 2016; Li 1996)
− Combined with genetically encoded tags	• Identify cellular location of a defined target without requiring antibodies	• Requires over-expression of protein of interest into the system	(Martell *et al.* 2017; Martell *et al.* 2012) (Shu *et al.* 2011) (Clarke & Royle 2018)
Flash-Freeze electron microscopy	• Record events with millisecond precision • Optogenetic stimulation allows precision activation of specific populations of cells	• Although high temporal resolution, it is not possible to follow events through time at the same synapse • Specialised equipment required	(Watanabe *et al.* 2018; Watanabe *et al.* 2013b; Borges-Merjane *et al.* 2020; Imig *et al.* 2020)
Uptake of fluorescent cargo	• Antibodies against specific SV proteins allows labelling of active synapses • Uptake of specific cargo can be used to selectively monitor specific pathways	• Generally only able to monitor one mode of endocytosis in isolation	(Kraszewski *et al.* 1995; Henkel *et al.* 2019) (Kirchhausen *et al.* 2008)

	• Possible to monitor live (in addition to fixed time points)		(Clayton *et al.* 2008; Cousin & Smillie 2021; Nicholson-Fish *et al.* 2015)
Labelling of the phospholipid membrane to follow SV recycling	• Membrane lipids are labelled and thus it is a non-biased method to monitor endocytosis pathways • Range of options covering the light spectrum facilitating multiplexing with other fluorescent markers • Monitor kinetics of uptake, recycling and release • pH sensitive dyes allowing sensitive detection of internalisation • Often compatible with super-resolution microscopy techniques	• Markers can suffer from photobleaching • Not specific to a particular internalisation pathway • Dye release rates are dependent on the departitioning kinetics	(Ryan 1999; Vanden Berghe & Klingauf 2006; Cheung *et al.* 2010) (Revelo *et al.* 2014; Gowrisankaran *et al.* 2020) (Collot *et al.* 2019) (Kahms & Klingauf 2018; Henkel *et al.* 2019)
pHluorin reporters (and red-shifted variants)	• Monitor multiple stages of the SV life cycle in the one experiment • Flexible experiment; easy to manipulate the external buffer to measure the total recycling pool or surface fraction or to isolate exocytosis • Quantitative measure of extent and kinetics in real-time • Potential to measure recycling in any acid compartment with pHluorin tagged to appropriate marker • Can be combined with other reporters to examine multiple features of presynaptic function simultaneously	• Require to transfect or transduce cells to express the reporter • Monitoring the recycling of a specific SV protein as a proxy for SV trafficking • Endocytic rate also has a component of reacidification	(Sankaranarayanan & Ryan 2000; McAdam *et al.* 2020; Harper *et al.* 2017; Soykan *et al.* 2017; Herman *et al.* 2018; Lazarevic *et al.* 2017; Jin *et al.* 2019; Li *et al.* 2017; Bae *et al.* 2020; Vaithianathan *et al.* 2019)

FRET/FLIM to measure protein-protein interactions	• Possible to determine if proteins interact in an intact cell • Measuring fluorescent life-time is independent of the fluorescent intensity and thus concentration of the reporter present and/or photobleaching	• Requires over-expression of reporter fluorescently tagged constructs with compatible excitation and emission spectra • Unless using more involved techniques, it is not possible to detect small changes to interactions	(Sakon & Weninger 2010; Verboogen *et al.* 2017; Stepien *et al.* 2019)
Super-resolution microscopy Single Molecule Localisation Microscopy	• Ability to localise fluorescent signals to less than 100 nm	• Require specialised fluorophores and/or microscopes to image samples • Low temporal resolution if reconstruction of an image is required	
− dSTORM	• Provides a resolution up to 10 nm in X and Y	• Can only be applied to fixed samples	(Dani *et al.* 2010; Klevanski *et al.* 2020; Lehmann *et al.* 2016)
− PALM	• High resolution images of protein localisations relative to sub-cellular structure • Ability to use method in fixed or live cells	• Require photo-activatable fluorophore tagged versions of protein of interest, which is typically over-expressed	(Bar-On *et al.* 2012; Pertsinidis *et al.* 2013; Smyth *et al.* 2013; Kasula *et al.* 2016)
− uPAINT	• Effective technique to track individual endogenous proteins	• Typically can only be used with membrane proteins with an extracellular domain	(Giannone *et al.* 2013; Choquet & Hosy 2020; Harper *et al.* 2020)
− sdTIM	• Monitor internalised proteins for example during endocytosis	• Currently limited to tracking a small number of particles/synaptic vesicles per nerve terminal	(Joensuu *et al.* 2017; Joensuu *et al.* 2016; Wang *et al.* 2016)

STED	• Specific processing of samples is not required • Can be carried out on fixed and live samples • Compatible with optical clearing methods	• Require specialised microscope to generate depletion beam	(Opazo & Rizzoli 2010; Willig *et al.* 2006; Westphal *et al.* 2008; Kamin *et al.* 2010; Hruska *et al.* 2018; Ke *et al.* 2016)
Expansion microscopy	• Do not require specialised microscope • Can use in combination with light sheet microscopy to image large regions of samples	• Relies on manipulation of samples in a gel • Can only be used on fixed samples	(Chang *et al.* 2017; Truckenbrodt *et al.* 2018)
Correlated light and EM microscopy	• Benefits of fluorescence microscopy to detect targets in live samples with high resolution cellular context information provided by EM	• Reliant on being able to detect the same target molecule post-fixation and processing for EM	(Mateos *et al.* 2018; Persoon *et al.* 2018; Shu *et al.* 2011; Watanabe *et al.* 2014a; Tao *et al.* 2018)
Mass spectrometry	• Identify unknown proteins with altered expression • Identify interaction partners • Detect post-translational modifications	• Dependent on the ability of the protein peptides to be detected in the mass spectrometer and the purity of samples generated	(Engholm-Keller *et al.* 2019; Fourneau *et al.* 2020)
− SILAC	• Easy to manipulate cell culture conditions to facilitate labelling of proteins under specific conditions or for specific timeframes • Allows calculation of protein turnover and half-lives	• Limited to cell culture	(Mathieson *et al.* 2018; Dörrbaum *et al.* 2018; Hakim *et al.* 2016)
− SILAM	• Label introduced in the diet of animal, allowing labelling of intact circuit	• Interpretations of the data can be complicated by the metabolism of the animal and potential re-use of labelled amino acids	(Hakim *et al.* 2016)

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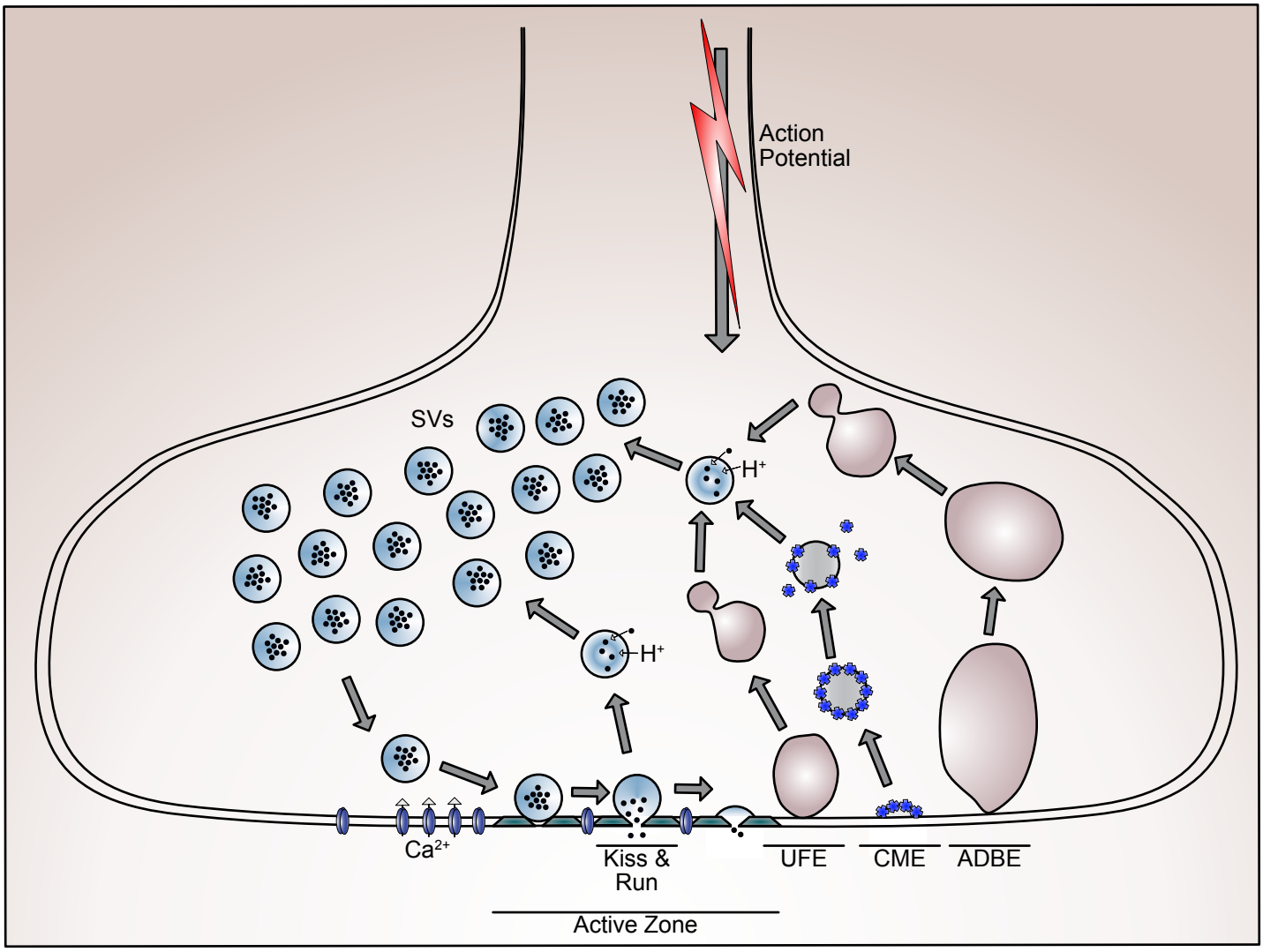
Figure 1 - Summary of the synaptic vesicle pathways at the presynapse



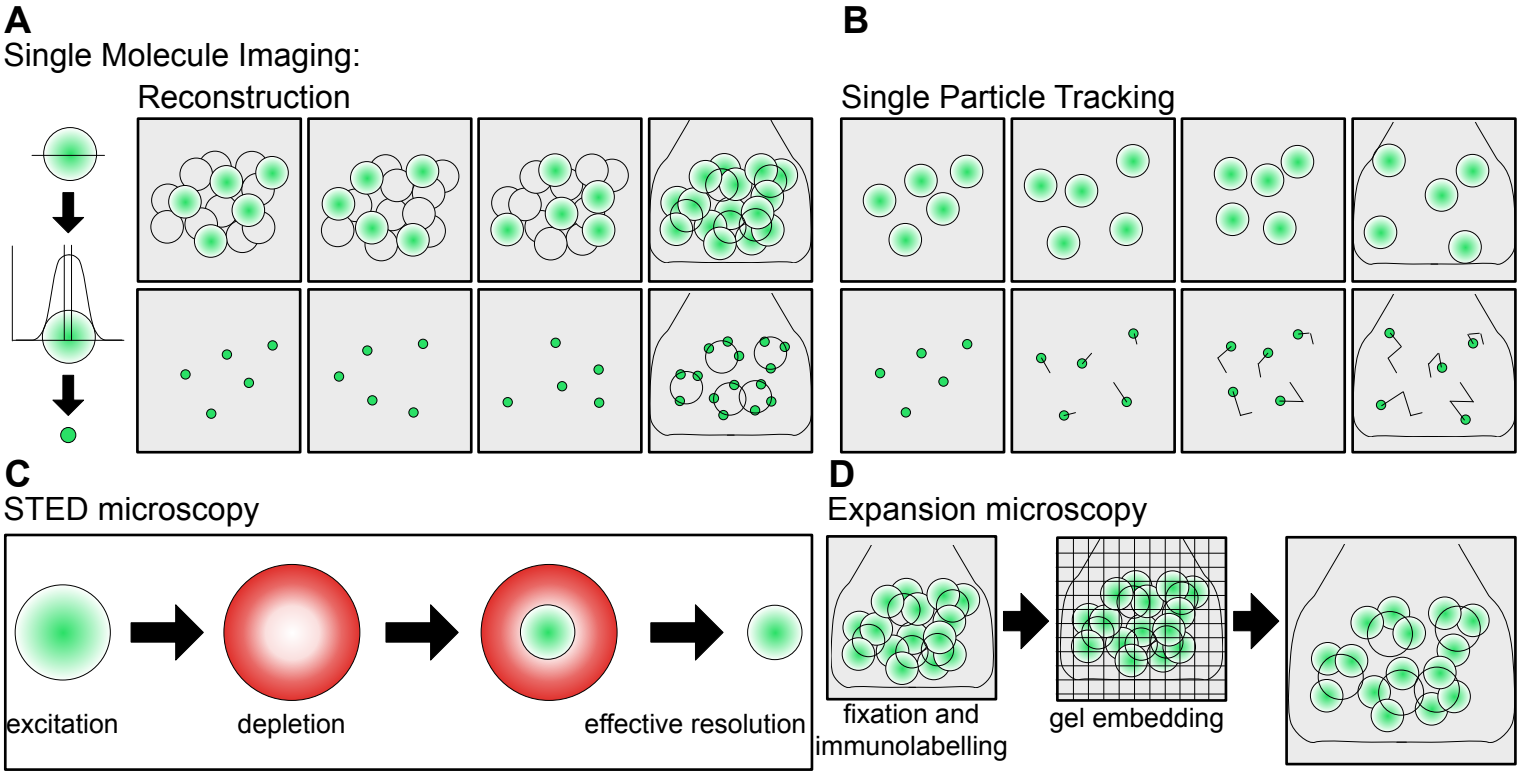


Figure 2 - Summary of super-resolution imaging techniques